

**Exposure of p19 matrix protein of human T-cell leukemia virus type I (HTLV-I) on the surface of MOLT-4#8 cells after virus adsorption**

**Brief Report**

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**Summary.** The p19 matrix (MA) protein of human T-cell leukemia virus type I (HTLV-I) was exposed on the surface of MOLT-4#8 cells in the very early step of the virus infection. Transfer of the virus-binding MOLT-4#8 cells from 4 °C to 37 °C resulted in increased detection of the viral gp46 and p19 MA protein on the cells, which was, however, inhibited by 4 °C or cytochalasin B treatment. These data showed that increased temperature and fluidity of the cell membrane were required for the increased detection of gp46 and p19 after viral adsorption. On the other hand, exposure of the p19 MA protein was not observed on the virus-treated U937 cells although gp46 was detected. This was not due to inefficient binding of the HTLV-I to the U937 cells, since the methanol-fixed cells were p19 MA protein-positive. MOLT-4#8 cells induced marked cell fusion when co-cultured with MT-2 cells, but U937 cells induced no fusion. All of these results indicated that these two cell lines differed in the property of plasma membrane in terms of degradation of HTLV-I envelope after viral adsorption. Uncoating of the HTLV-I might occur on the plasma membrane, especially on MOLT-4#8 cells.

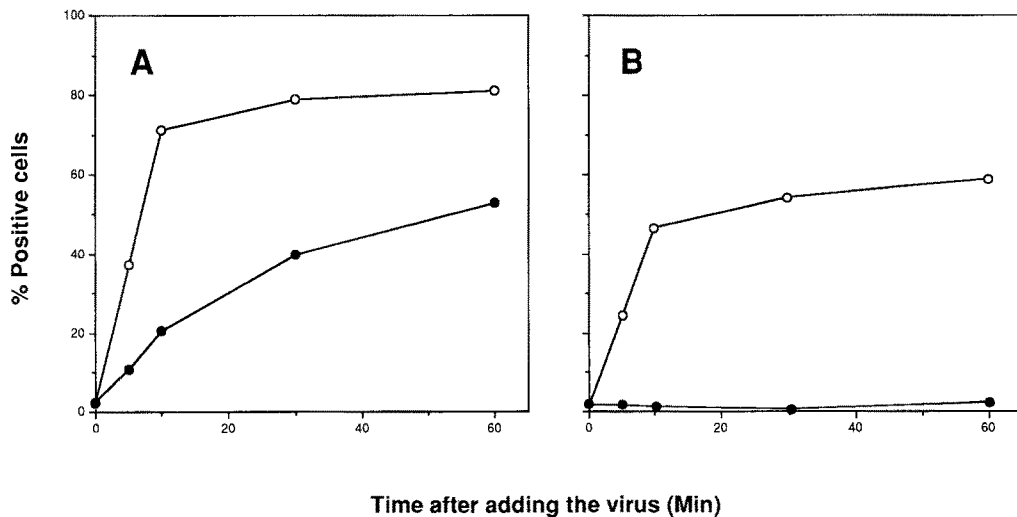
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Human T-cell leukemia virus type I (HTLV-I) is known to be the etiologic agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2, 3, 8, 9, 14]. In vivo, ATL shows a preferential incidence in CD4<sup>+</sup> lymphocytes [11]. However, the cellular receptor(s) for HTLV-I is (are) seemingly distinct from the CD4 molecule [10, 4]. HTLV-I is usually transmitted to other non-infected cells by

co-culture of the virus-carrying cells with peripheral blood lymphocytes from healthy donors [7, 13]. Because of the inability of cell-free HTLV-I to infect cells, little is known about the viral receptor and early steps of virus-infection such as adsorption, penetration and uncoating.

Interest in early virus-cell interactions is rapidly growing. Previously, we utilized HTLV-I concentrated from supernatants of MT-2 cells (an HTLV-I-transformed and virus producer T cell line) and monoclonal antibodies (mAbs) to gp 46 (env) or p19 (gag) to assess HTLV-I adsorption on various target cells [1, 5]. MOLT-4#8 and U937 cells were shown to specifically adsorb high levels of HTLV-I. However, striking differences were found: (i) MOLT-4#8 cells showed marked cell-fusion after co-cultivation with MT-2 cells, whereas U937 cells did not; (ii) HTLV-I-adsorbed MOLT-4#8 cells exhibited antigen-positivity for both gp 46 and p19, while the virus-adsorbed U937 cells were only gp 46-positive. Based on these observations, we hypothesized that matrix (MA) gag protein (p19) of HTLV-I may be exposed during the post-binding steps on the plasma membrane of MOLT-4#8 cells but not on U937 cells.

For detection of gp46 (env) and p19 (MA gag) on the plasma membrane after adsorption of cell-free HTLV-I, flow cytometric analyses using mAbs to gp 46 (REY-7) and p19 (GIN-14) were applied, as previously described [1]. In brief, MOLT-4#8 or U937 cells were treated with cell-free HTLV-I at 37 °C for various times as indicated in Fig. 1 and washed with chilled washing medium containing 2% fetal calf serum (FCS) in RPMI 1640 medium. Then, the cells were mixed with rat mAb against gp 46 (REY-7) or mouse mAb to p19 (GIN-14) at 4 °C for 40 min. As a negative control, MOLT-4#8 or U937 cells were incubated with the mAbs followed by the FITC-conjugated IgG under the same conditions. The cells were again washed and stained with fluorescein isothio-



**Fig. 1.** Time course of gp46 (○) and p19(●)-positive cell detection in HTLV-I-treated MOLT-4#8 cells (A) and U937 cells (B). REY-7 and GIN-14 mAbs were used to detect gp 46 and p19 positive cells, respectively

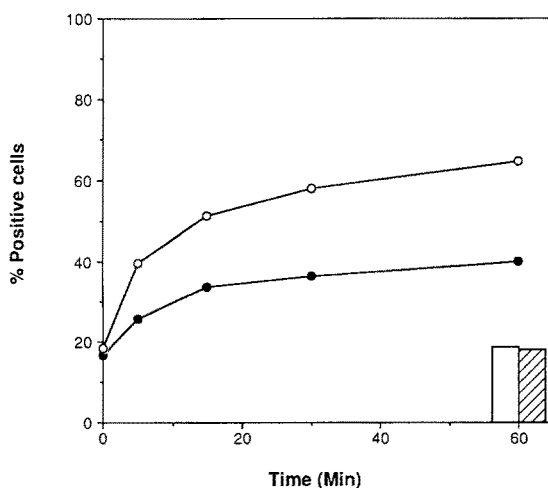
cyanate (FITC)-conjugated anti-rat or -mouse IgG at 4°C for 40 min. The stained cells were then washed, fixed with 1% formaldehyde in phosphate-buffered saline (PBS) and analyzed for fluorescence intensity on FACScan (Becton & Dickinson). Positivity was measured as % positive cells as well as intensity of fluorescence. Both parameters were parallel in all experiments.

Figure 1 shows % positive cells of MOLT-4#8 (panel A) and U937 (panel B) cells after adding the HTLV-I. When the virus-adsorbed MOLT-4#8 cells were treated with REY-7 mAb, nearly 40% of the cells were detected as positive 5 min after incubation at 37°C (Fig. 1A). After 30 min, 80% positive cells were observed. When the U937 cells were stained for surface expression of gp 46 env protein (Fig. 1B), the kinetics of positive cell-increase with time was similar to that of MOLT-4#8 cells. Maximum positivity was, however, about 60%. When MOLT-4#8 cells were stained with GIN-14 mAb, the increase in % positive cells was slower than that in the case of REY-7 mAb staining (Fig. 1A). Fifty % of the cells were positive for p19 MA-protein 60 min after inoculation of the virus. However, the U937 cells stained with GIN-14 mAb were mostly negative even after 60 min incubation (Fig. 1B). These results confirm the inability of U937 cells to present p19 MA protein on their surfaces in the post-adsorption step. However, after inoculation of HTLV-I to MOLT-4#8 cells, not only env gp 46 but also p19 proteins were detected on the membrane and the positivity of both proteins was increased with time when incubated at 37°C. These data suggest that enzymatic breakdown of the intact virus or de novo synthesis of gag proteins might occur in MOLT-4#8 cells. To exclude the possibility that p19 MA protein was expressed by de novo protein synthesis after the viral inoculation, MOLT-4#8 cells were pretreated with cycloheximide (0.1 and 1 µg/ml) or puromycin (5 and 50 µg/ml) as translational inhibitors and then the virus was added. The rate of FITC-positivity was not affected by the cycloheximide or puromycin pretreatment in either env or gag expression (Table 1).

Next, we examined the temperature effect on detection of the viral gag or env proteins on the cell surface (Fig. 2). MOLT-4#8 cells were treated with the concentrated virus at 4°C for 30 min, washed twice and resuspended with warm

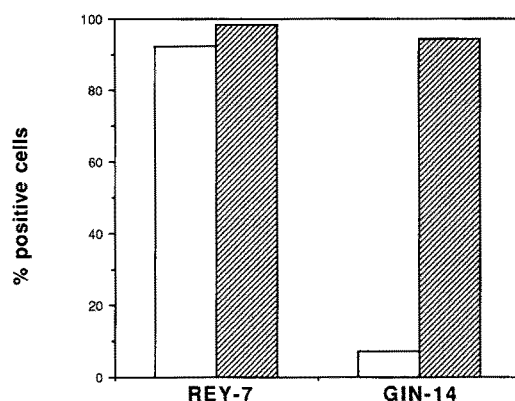
**Table 1.** Effect of cycloheximide and puromycin on gp 46 and p19 positivity of HTLV-I-treated MOLT-4#8 cells

Pretreatment of MOLT-4#8 cells with	Dose	% Positive cells stained by	
		REY-7	GIN-14
None		84.0	61.0
Cycloheximide	0.1 µg/ml	88.9	67.0
	1 µg/ml	85.0	67.3
Puromycin	5 µg/ml	84.8	56.2
	50 µg/ml	86.8	58.1



**Fig. 2.** Effect of temperature shift ( $4^{\circ}\text{C} \rightarrow 37^{\circ}\text{C}$ ) and cytochalasin B on gp46 ( $\circ$ ) and p19 ( $\bullet$ ) positive cells. Open and hatched bars indicate % positive cells in gp46 and p19, respectively, when the virus-adsorbed MOLT-4#8 cells were treated with  $10\ \mu\text{g/ml}$  of cytochalasin B at  $37^{\circ}\text{C}$  for 60 min

washing medium. The cells were then transferred to  $37^{\circ}\text{C}$ . Incubation of the cells at  $4^{\circ}\text{C}$  for 30 min induced only 20% positive cells, as compared with 80% and 40% in gp46 and p19 positive cells, respectively, at  $37^{\circ}\text{C}$  for 30 min (Fig. 1A). The incubation at  $37^{\circ}\text{C}$  was terminated at 5, 15, 30 and 60 min by adding cold washing medium. The cells were stained as described above. In terms of viral gp46 expression, a temperature shift from  $4^{\circ}\text{C}$  to  $37^{\circ}\text{C}$  resulted in a gradual increase of positive cells from 18% to 65% (Fig. 2). When mAb to p19 was used, a similar increase was observed, albeit maximum positivity was 40% after 60 min. Exactly the same pattern of increase was obtained by assessing the fluorescence intensity instead of % positive cells. Next, MOLT-4#8 cells adsorbed with HTLV-I at  $4^{\circ}\text{C}$  for 30 min and washed were treated with cytochalasin B ( $10\ \mu\text{g/ml}$ ) and incubated at  $37^{\circ}\text{C}$  for 60 min. Cytochalasin B is known to affect the fluidity of cell membrane by inhibiting the polymerization of cytoskeletal actin filaments. Cytochalasin B treatment of the virus-binding MOLT-4#8 cells completely inhibited the increase of the FITC-positive cells. About 20% of the cells were positive for both env and gag even after 60 min incubation at  $37^{\circ}\text{C}$  as shown in Fig. 2. Thus, the increase in positivity of viral gag and env proteins on HTLV-I-adsorbed MOLT-4#8 cells depended on the temperature and the membrane fluidity. We can speculate that the envelope protein of HTLV-I may be torn on the MOLT-4#8 cell membrane resulting in exposure of the inner matrix protein p19. Because of the cell membrane fluidity, fragments (or antigen-epitopes) of env and gag proteins might disperse, which could become more detectable, increasing the fluorescence intensity and % positive cells. If this is the case, HTLV-I virion may stay intact on the U937 cell membrane probably due to the lack of some proteolytic enzyme (s) and/or

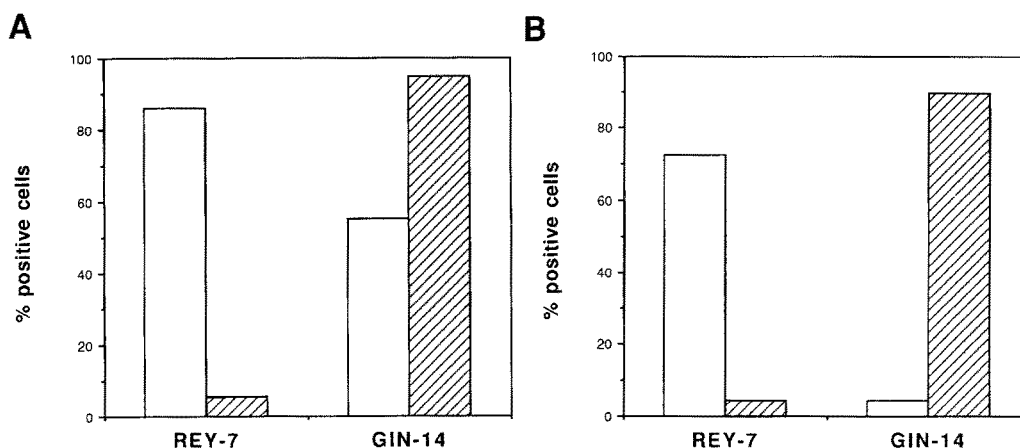


**Fig. 3.** Effect of methanol fixation on gp46 and p19 positive cells in unfixed (open bar) and methanol-fixed (hatched bar) MT-2 cells. As a negative control, the MT-2 cells were stained without mAbs

different degree of membrane fluidity. Thus, we could not detect the p19 MA protein on HTLV-I-adsorbed U937 cells.

To confirm that the U937 cells treated with HTLV-I can actually adsorb the intact viral particles containing the p19 MA protein, we examined the effect of methanol fixation on the detection of viral proteins in the HTLV-I-infected cells. MT-2 cells or the virus-adsorbed cells at 37 °C for 30 min were washed and treated with 70% methanol at 4 °C for 3 min. The cells were washed again and stained by REY-7 or GIN-14 as described. First, as a control, MT-2 cells were examined (Fig. 3). Without methanol treatment, gp46 was strongly detected but p19 MA protein was only weakly detectable on the surface of MT-2 cells. After methanol fixation, more than 90% in both gp46 and p19 became positive (Fig. 3). Methanol fixation of the cells could allow antibodies to penetrate the membrane into the cells. Figure 4A displays that methanol fixation of the HTLV-I-adsorbed MOLT-4#8 cells decreased the gp46-positive cells from 85% to 5% but increased p19-positive cells from 55% to 95%. In the case of the virus-treated U937 cells (Fig. 4B), p19 MA protein was clearly detected in the fixed cells but was almost undetectable on the unfixed U937 cells. These data indicated that the intact virus was truly present on the U937 cells but p19 MA protein was not exposed on the surface of the unfixed cells. Fluorescence positivity of gp46 decreased to the back-ground level by methanol treatment in both U937 and MOLT-4#8 cells (Fig. 4A and B) probably due to lysis out of viral envelope glycoprotein induced by the treatment.

We have studied the early steps of HTLV-I infection to the target cells especially on the plasma membrane. Previously, we demonstrated that co-culturing MT-2 cells with MOLT-4#8 cells led to syncytium-formation but did not with U937 cells [5]. By cell-free HTLV-I adsorption, gp46 was detectable on the surface of both types of cell, but p19 MA protein was positive on MOLT-4#8 cells only. These data prompted us to compare these two types of cells in terms of the properties of the plasma membrane after the viral



**Fig. 4.** Effect of methanol fixation on gp46 and p19 positive cells in HTLV-I-adsorbed MOLT-4#8 cells (A) and U937 cells (B). REY-7 and GIN-14 mAbs were used to detect gp46 and p19 positive cells, respectively. Open and hatched bars represent unfixed and methanol-fixed cells, respectively

adsorption. Our data suggest that p19 MA protein may be exposed on the cell membrane and that the increased fluorescence-intensity and positivity after 37°C incubation may be due to the spread of the envelope fragments by membrane fluidity. Lysis of viral envelope on the cell membrane might be necessary for the next steps of HTLV-I infection such as fusion and penetration. In fact, U937 cells induce no fusion when co-cultured with MT-2 cells. This finding recalls the case of human immunodeficiency virus type 1 (HIV-1), in which the V3 region of envelope gp120 is cleaved on cell membrane by proteolysis [12]. In the cases of orthomyxoviruses, paramyxoviruses and coronaviruses, activating proteolytic cleavages catalyzed by cellular proteases late in the secretory pathway are critical for the fusion activity [6]. In this regard, we have used several available anti-protease agents (amastatin, phosphoramidon, E-64, arphamenine A, leupeptin, antipain, and pepstatin A) to block the exposure of p19 MA protein on the virus-adsorbed MOLT-4#8 cells. However, no effective agent has yet been found. It remains to be elucidated whether the exposure of p19 MA protein is followed by successive uncoating of the virus. Further studies are needed to identify the mechanism involved.

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### References

1. Akari H, Kannagi M, Shinjo T, Harada S (1993) Simple assay system for detecting Human T-cell leukemia virus type I (HTLV-I)-binding cells and its application in titrating binding inhibitory antibodies. *Lab Invest* 69: 629–634

2. Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A (1985) Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* ii: 407–409
3. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita K, Shirakawa S, Miyoshi I (1981) Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc Natl Acad Sci USA* 78: 6476–6480
4. Kannagi M, Kuroda MJ, Maeda Y, Harada S (1991) Coexistence of fusion receptors for human T-cell leukemia virus type-I (HTLV-I) and human immunodeficiency virus type-I (HIV-I) on MOLT-4 cells. *Microbiol Immunol* 35: 729–740
5. Kuroda MJ, Kannagi M, Masuda T, Harada S (1992) Inefficient transmission of HTLV-I to MOLT-4 cells by cell-free virus and cocultivation. *Intervirology* 34: 202–212
6. Marsh M, Helenius A (1989) Virus entry into animal cells. *Adv Virus Res* 36: 107–151
7. Miyoshi I, Kubonishi I, Yoshimoto S, Akagi T, Ohtsuki Y, Shiraishi Y, Nagata K, Hinuma Y (1981) Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature* 294: 770–777
8. Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M (1986) HTLV-I associated myelopathy, a new clinical entity. *Lancet* i: 1031–1032
9. Popovic M, Reitz Jr MS, Sarngadharan MG, Robert-Guroff M, Kalyanaraman VS, Nakao Y, Miyoshi I, Minowada J, Yoshida M, Ito Y, Gallo RC (1982) The virus of Japanese adult T-cell leukemia is a member of the human T-cell leukemia virus group. *Nature* 300: 63–66
10. Sommerfelt MJ, Williams BP, Clapham PR, Solomon E, Goodfellow PN, Weiss RA (1988) Human T cell leukemia viruses use a receptor determined by human chromosome 17. *Science* 242: 1557–1559
11. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H (1977) Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood* 50: 481–492
12. Werner A, Levy JA (1992) Human immunodeficiency virus type 1 envelope gp120 is cleaved after incubation with recombinant soluble CD4. *J Virol* 67: 2555–2574
13. Yamamoto N, Okada M, Koyanagi Y, Kannagi M, Hinuma Y (1982) Transformation of human leukocytes by cocultivation with an adult T cell leukemia virus producer cell line. *Science* 217: 60–62
14. Yoshida M, Miyoshi I, Hinuma Y (1982) Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* 79: 2031–2035

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